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Immunoglobulin variable region sequences of two human monoclonal antibodies directed to an onco-developmental carbohydrate antigen, lactotetraosylceramide(LcOse[4]Cer)( Dissertation\_全文 )

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(癌胎児性糖鎖抗原ラクトテトラオシルセラミド (LcOse<sub>4</sub>Cer) に対する、2種のヒト型モノクローナル抗体の免疫グロブリン可変領域のシーケンス)

Immunoglobulin variable region sequences of two human monoclonal antibodies directed to an onco-developmental carbohydrate antigen, lactotetraosylceramide (LcOse<sub>4</sub>Cer) (癌胎児性糖鎖抗原ラクトテトラオシルセラミド (LcOse<sub>4</sub>Cer) に対する、2種のヒト型モノクローナル抗体の免疫グロブリン可変領域のシーケンス)

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IMMUNOGLOBULIN VARIABLE REGION SEQUENCES OF TWO HUMAN  
MONOCLONAL ANTIBODIES DIRECTED TO AN ONCO-DEVELOPMENTAL  
CARBOHYDRATE ANTIGEN, LACTOTETRAOSYLCERAMIDE (LcOse4Cer)\*

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Running title: Variable region sequences of human anti-Lc4 mAbs

Footnote

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\*\*\*The nucleotide sequences reported in this paper have been submitted to the  
EMBL Data Bank with accession numbers X72744, X72745, X72746 and X72747.

Abbreviations used: Lc4, lactotetraosylceramide, LcOse4Cer (Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$ 1 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4Glc $\beta$ 1 $\rightarrow$ 1Cer); SSEA-1, stage-specific embryonic antigen-1; TLC, thin-layer chromatography; Id, idiotype; FR, the framework region of antibody; CDR, the complementarity-determining-region of antibody. Neutral glycolipids were abbreviated according to recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1978). Gangliosides were abbreviated according to the system of Svennerholm (1964).



# ABSTRACT

A human monoclonal antibody, 11-50, was generated and was shown to recognize an onco-developmental carbohydrate antigen, LcOse4Cer. The isotype of this antibody was IgM,  $\lambda$ , similar to the previously known human anti-LcOse4 antibodies, such as IgM<sup>WOO</sup> and HMST-1. We raised a murine anti-idiotypic antibody G3 (IgG1,  $\kappa$ ) against 11-50, and tested its reactivity towards the affinity purified human polyclonal anti-LcOse4 antibodies prepared from pooled human sera using a Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$ -immobilized column. The results indicated that at least a part of the human polyclonal anti-LcOse4 antibodies shared the G3 idiotype with 11-50. We further analyzed the sequence of variable regions of the two anti-LcOse4 antibodies, 11-50 and HMST-1. Sequence analysis of the heavy chain variable regions indicated that the V<sub>H</sub> regions of these two antibodies were highly homologous to each other (93.5% at the nucleic acid level), and these antibodies utilized the germline genes V<sub>H</sub>1.9III and hv3005f3 as the V<sub>H</sub> segments, which are closely related germline genes of the V<sub>H</sub>III family. It was noted that these germline V<sub>H</sub> genes are frequently utilized in fetal B cells. The J<sub>H</sub> region of both antibodies was encoded by the J<sub>H</sub>4 gene. For the light chain, the V $\lambda$  segments of the two antibodies were 96.3% homologous to each other at the nucleic acid level. The V $\lambda$  segments of both antibodies showed the highest homology to the rearranged V $\lambda$  gene called V $\lambda$ II.DS among reported V $\lambda$  genes, while the exact germline V $\lambda$  genes encoding the two antibodies were not yet registered in available sequence data banks. The amino acid sequences of the J $\lambda$  segments of both antibodies were identical. These results indicate that the two human antibodies recognizing the onco-developmental carbohydrate antigen Lc4 are encoded by the same or very homologous germline genes.

## INTRODUCTION

LcOse4Cer (Lc4, lactotetraosylceramide) is a type 1 chain carbohydrate antigen, which is a precursor substance for the synthesis of Lewis blood type antigens, such as Le<sup>a</sup> and Le<sup>b</sup>, and also for the synthesis of cancer-associated antigens, such as CA19-9 and CA50. Two murine anti-Lc4 monoclonal antibodies have been found among the antibodies raised against human teratocarcinoma cells (Gooi *et al.*, 1983; Rettig *et al.*, 1985; Fukuda *et al.*, 1986). The distribution of the antigen in human embryonic tissues as well as in cancer tissues has been studied extensively using these antibodies (Gooi *et al.*, 1983; Rettig *et al.*, 1985; Williams *et al.*, 1982). The antigen is reported to be detectable in meconium and in the epithelial cells of the fetal bronchus and intestine (Williams *et al.*, 1982; Karlsson and Larson, 1979). Furthermore, the antigen is expressed on the surface of a variety of human cancers including embryonal carcinoma, teratocarcinoma, renal cell carcinoma, adenocarcinoma of the gastro-intestinal tract and cancers of the uterine cervix (Rettig *et al.*, 1985; Williams *et al.*, 1982; Nozawa *et al.*, 1989; Tsukazaki *et al.*, 1991). In this sense, some investigators consider Lc4 to be an onco-developmental antigen. The antigen is expressed in a few normal adult tissues such as some epithelial cells of the gastrointestinal tract, uterine cervical glandular cells and renal tubular cells (Rettig *et al.*, 1985; Williams *et al.*, 1982; Nozawa *et al.*, 1989; Tsukazaki *et al.*, 1991).

An interesting aspect of the Lc4 antigen is that antibodies against this antigen have been frequently found in the sera of humans. Anti-Lc4 antibodies are known to occur sometimes in patients with motor neuron diseases (Latov *et al.*, 1988; Ito and Latov, 1988). A monoclonal paraprotein directed to Lc4 was reported to be present in the serum of a patient with lung cancer (IgM<sup>WOO</sup>) (Kabat *et al.*, 1982). The latter case was of particular interest, since the favorable clinical course of the patient is possibly explained by the presence of this antibody in his serum. The human monoclonal antibody directed to this antigen has not infrequently been established; in addition to IgM<sup>WOO</sup>, a human anti-LcOse4 antibody was established from the



lymphocytes prepared from a patient with uterine endometrial cancer (Nozawa *et al.*, 1989), and we also obtained a human monoclonal antibody directed to this antigen (this paper).

Recently, we and other workers have examined the molecular structure of the variable regions of murine monoclonal antibodies against onco-developmental carbohydrate antigens and found restricted usage of  $V_H$  and  $V_L$  against SSEA-1 and its related antigens (Zenita *et al.*, 1988; Kimura *et al.*, 1989; Snyder *et al.*, 1990). A similar finding is also reported with regard to human anti-carbohydrate antibodies; the anti-I antibodies which accumulate in the sera of patients with cold agglutinin disease were recently shown to be encoded by a highly homologous series of  $V_H$  genes (Leoni *et al.*, 1991; Pascual *et al.*, 1991). We report here the nucleic and derived amino acid sequences of the immunoglobulin variable regions of the two human monoclonal antibodies recognizing Lc4.

## MATERIALS AND METHODS

### *Generation of hybridoma cells*

The heterohybridoma 11-50 (IgM,  $\lambda$ ) was obtained according to the method described by Köhler and Milstein (1975). Briefly, human lymphocytes isolated from a surgically resected tonsil were fused with hypoxanthine-aminopterin-thymidine-sensitive murine fusion partner cells (P3/X63-Ag8U1) at a ratio of 2:1 using polyethylene glycol. The glycolipid mixture prepared from A375 cells was used as the antigen in ELISA of culture supernatants of hybridomas in the cloning procedures. Heterohybridoma HMST-1 (IgM,  $\lambda$ ) was obtained as previously described (Nozawa *et al.*, 1989).

G3, an anti-idiotypic antibody against 11-50, was established according to the previously described methods (Hirashima *et al.*, 1990). BALB/c mice were immunized at day 0, day 7 and day 28, with 100  $\mu$ g of the 11-50 antibody which was

conjugated with KLH. The spleen cells were fused with mouse myeloma cells P3/X63-Ag8U1 (P3U1) on day 31. The antibody 11-50 was used as the immobilized antigen in ELISA of culture supernatants of hybridomas for the cloning procedures. The isotype of the G3 antibody was IgG<sub>1</sub>,  $\kappa$ . To test the inhibitory activity of G3 to the binding of 11-50 with the original antigen, Lc<sub>4</sub> was immobilized at the bottom of the wells of the 96-well plate. To this, G3 and biotinylated 11-50 were added, followed by the addition of avidin-peroxidase (2  $\mu$ g/ml) and substrate solution (0-phenylenediamine).

#### *Cultured Cells and Preparation of Standard Glycolipids.*

Human melanoma cells (A375), teratocarcinoma cells (2102Ep), colon carcinoma cells (LS174T, SW80, SW48, HT29, BM314), breast cancer cells (T47D) and lung cancer cells (KNS62) were cultured in Dulbecco's modified MEM supplemented with 10% fetal calf serum. For the preparation of glycolipids from these cells, tumors xenografted to nude mice were used as starting material. Glycolipids were prepared from these tumor tissues by the method described earlier (Hakomori and Kannagi, 1986). Briefly, total lipids were extracted from those tissues with isopropanol/ hexane/ water (55:20:25, v/v/v) and partitioned according to Folch. The glycolipids in the lower layer fraction of the Folch partition were further purified by acetylation followed by Florisil column chromatography, and subjected to DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) column chromatography. The neutral glycolipids were eluted with chloroform/ methanol/ water (30:60:8, v/v/v, fraction A) and gangliosides, were then eluted with chloroform/ methanol/ 0.8N sodium acetate (30:60:8, v/v/v, fraction B). In some experiments, neutral glycolipids in the fraction A were further separated by preparative TLC using the solvent system; chloroform/ methanol/ water (60:35:8, v/v/v).

Pure Lc<sub>4</sub> was prepared by defucosylation of Le<sup>a</sup> which was prepared from the neutral glycolipid fraction of either human meconium or cultured colon cancer cells (Karlsson and Larson, 1979; Iwamori *et al.*, 1988). GM<sub>1</sub>, GM<sub>2</sub> and GM<sub>3</sub> were



purchased from Supelco Inc. (Bellfonte, PA). Asialo-GM1 (Gg4) and asialo-GM2 (Gg3) were prepared by mild acid hydrolysis of GM1 and GM2 gangliosides, respectively (Taki *et al.*, 1984; Shigeta *et al.*, 1987). SSEA-3 (IV<sup>3</sup>Gal $\beta$ Gb4) was purified from human teratocarcinoma cell line, 2102Ep, as described previously (Kannagi *et al.*, 1983a; Kannagi *et al.*, 1983b). Paragloboside (nLc4) was prepared by preparative TLC of acidic glycolipid fractions of human erythrocytes which had been mildly acid-hydrolyzed. Amino-CTH (Lc3) was prepared by hydrolysis of paragloboside using Jack bean  $\beta$ -galactosidase (Sigma, St. Louis, MO). The IV<sup>2</sup>Fuc $\alpha$ nLc4 (H1-glycolipid) and IV<sup>3</sup>Gal $\beta$ nLc4 (Kraus-glycolipid) were purified from the same source as described earlier (Hakomori *et al.*, 1986).

#### *Assessment of the specificity of mAbs toward various glycolipids*

TLC-immunostaining was performed using a HPTLC plate (Si-HPF plate 7011-3, J. I. Baker Chemical Co., Phillipsburg, NJ) and <sup>125</sup>I-protein A (Du pont, Boston, MA) as described first by Magnani *et al.* (1980) and subsequently modified by us (Kannagi *et al.*, 1982). ELISA was performed using glycolipid antigens immobilized at the bottom of 96-well plates by a standard method described previously (Hakomori *et al.*, 1986). Peroxidase-conjugated goat anti-human IgM ( $\mu$ -chain specific) and anti-human IgG ( $\gamma$ -chain specific) antibodies were obtained from Cappel Inc. (Malvern, PA).

#### *Detection of idiotype positive component in pooled normal human sera*

Pooled normal human sera were applied to a Synsorb affinity column on which Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$  is immobilized (Chembiomed LTD, Alberta, Canada). Human pooled sera which had been x 5 diluted with PBS were subjected to the column. After extensive washing with 0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, the adsorbed antibodies were eluted with 0.2 M glycine-HCl, pH 2.3. The eluates were neutralized with 1.0 M Tris-HCl, pH 8.5, and the titer of the 11-50-like antibody in each fraction was measured by ELISA using a combination of the immobilized neutral glycolipids

from LS174T cells which contains Lc4 and the biotinylated anti-idiotypic antibody G3. First, the neutral glycolipids of LS174T cells was immobilized at the bottom of each well of the 96 well plates, and, after blocking, they were reacted with fractionated human serum samples. To this, the biotinylated G3 antibody, avidin-peroxidase, and substrate solution were added sequentially. The titer of the G3-idiotype-positive anti-Lc4 antibody was measured in this assay system. One unit is defined as absorbance at 500 nm when 11-50 (1 µg/ml) was assayed.

#### *Sequencing of immunoglobulin heavy and light chain variable region genes*

Total RNA was prepared from  $1 \times 10^8$  cells using the guanidine isothiocyanate method. Poly (A)<sup>+</sup> RNA was isolated using Oligotex dT30 (Takara Inc., Kyoto, Japan). Direct sequencing of mRNA of immunoglobulin heavy and light chain variable region genes was performed using the dideoxy chain termination method (Geliebter *et al.*, 1986). The 5'-end of the primers was labeled with <sup>32</sup>P. The mixture of the primer and mRNA was heated at 100°C for 2 min and cooled to room temperature for 30 min. Avian myeloblastosis virus reverse transcriptase (Life Sciences, St Petersburg, FL), deoxy- and dideoxy-NTPs (Takara Inc., Kyoto, Japan) were added to the annealing mixture, and incubated at 48°C for 60 min. The cDNA fragments were separated by electrophoresis using 6% or 8% polyacrylamide urea gels, and detected by autoradiography. Partial sequences of variable regions were obtained using the universal oligonucleotide primers, which were 5'ACGCTGCTCGTATCCGAC3' for the C $\mu$  gene and 5'CAGTGTGGCCTTGTTGGCTTG3' for the C $\lambda$  gene. Primers for further extension were synthesized according to the obtained partial sequence data; 5'CGTGTTCCTTGAATTGTC3' for the V<sub>H</sub> gene and 5'AGGCCGTGTTGCCAGACTT3' for the V $\lambda$  gene. Sequencing was performed as described above and the total sequences of the coding regions were obtained.

In order to confirm the V region sequences obtained by mRNA sequencing, RT-PCR was performed to amplify the rearranged immunoglobulin variable regions. The first-strand cDNA was synthesized from 20 µg of total RNA with oligo d(T)



primer and avian myelomatosis virus reverse transcriptase. The primers for PCR were 5'CAGCTGCAGCTGGTGGAG3' for the 5'-end of the V<sub>H</sub> segment, 5'TCTGCCCTGACTCAGCC3' for the 5'-end of the V<sub>λ</sub> segment. The same universal constant region primers, as had been used in the mRNA sequencing, were used for the 3'-end of the segments. Second strand synthesis and amplification were carried out via PCR on one fourth of the first-strand synthesis reaction mixture, 25 pmol of primers, 200 μM each of the dNTP, 10 mM Tris-HCl, pH 8.3 at 37 °C, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, and 2.5 U of *Thermus aquaticus* (*Taq*) thermostable DNA polymerase (Cetus, Emeryville, CA). Thirty cycles of amplification were performed using a Perkin Elmer Cetus DNA thermal cycler. Each cycle consisted of 1 min. at 94 °C (denaturation), 1 min. at 50 °C (annealing), and 2 min. at 72 °C (extension). The amplified DNA was separated on 5% polyacrylamide gel, and the band of the expected size was excised. The DNA fragments recovered from the gel were blunt-ligated into the phosphatase-treated *Hinc* II site of M13mp19(+) vector, and nucleotide sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical Co., Cleveland, OH). To avoid amplification artifacts by *Taq* polymerase, at least three clones were sequenced.

## RESULTS

### *Carbohydrate specificities of the human anti-Lc4 antibodies 11-50 and HMST-1*

In our preliminary experiments, several cultured human cancer cell lines, including melanoma cells (A375), colon cancer cells (LS174T, SW80, SW48, HT29, BM314), breast cancer cells (T47D) and lung cancer cells (KNS62) were found to express the antigen which was recognized by the 11-50 antibody.

When the neutral glycolipids extracted from LS174T cells were tested for reactivity with the 11-50 antibody by TLC-immunostaining technique, a clear positive band was identified as shown in Fig. 1, Lane 2. The LS174T cells contained a large amount of the reactive glycolipid. Comparing the TLC mobilities of various

Fig.



glycolipids as seen in the orcinol/H<sub>2</sub>SO<sub>4</sub> staining (Fig. 1, Lane 1), the reactive glycolipid was thought to be a glycolipid having 3 to 5 sugar residues. The TLC mobility of the reactive glycolipid was identical to the mobility of the glycolipid band detected with the HMST-1 antibody (Fig. 1, Lane 3), which is reported to be reactive with Lc4.

ELISA using a panel of various standard glycolipids was performed, to determine whether the glycolipid recognized by 11-50 is Lc4. The 11-50 antibody strongly reacted with Lc4 having Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$  terminus (Fig. 2). It also reacted weakly with asialo-GM1 (Gg4) and SSEA-3 (IV3Gal $\beta$ Gb4), both of which have the Gal $\beta$ 1 $\rightarrow$ 3GalNAc $\beta$  terminus. No reactivity was observed with nLc4 (paragloboside, having the Gal $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$  terminus), asialo-GM2 (Gg3, having the GalNAc $\beta$ 1 $\rightarrow$ 4Gal $\beta$  terminus), amino-CTH (Lc3, having the GlcNAc $\beta$ 1 $\rightarrow$ 3Gal $\beta$  terminus), GM3 (having the NeuAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$  terminus), IV<sup>2</sup>Fuc $\alpha$ nLc4 (H1-glycolipid) or IV<sup>3</sup>Gal $\beta$ nLc4 (Kraus-glycolipid). These results indicate that the carbohydrate structure recognized by 11-50 is Lc4 having the Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$  terminus, and the antibody is slightly cross-reactive with other glycolipids having the Gal $\beta$ 1 $\rightarrow$ 3GalNAc $\beta$  terminus. The result of TLC-immunostaining using these purified glycolipid standards also confirmed that Lc4 was the only reactive glycolipid (data not shown). The pure Lc4 antigen oligosaccharide (lactotetraose) completely inhibit the reaction of 11-50 antibody with A375 and LS174T cells at the concentration of  $7.2 \times 10^{-4}$  M, which also confirmed that Lc4 is the cell surface antigen recognized by the 11-50 antibody.

Fig. 2

Fig. 3 compares the reactivity of 11-50 and HMST-1 toward purified Lc4 antigen. Both antibodies react with Lc4, and 11-50 seems to be a higher affinity antibody than HMST-1. Both antibodies were secreted as pentameric IgM, as judged from the elution profiles of the gel filtration chromatographies during the purification procedures of both antibodies.

Fig. 3

*Detection of idiotype positive component in pooled normal human sera*

The anti-Id antibody G3, raised against the 11-50 antibody had a strong binding activity to the 11-50 antibody, and had no reactivity against four other control human monoclonal antibodies (all IgM, including three IgM,  $\kappa$  and one IgM,  $\lambda$ ). The G3 antibody did not react even with HMST-1, and was tentatively assumed to be specific to a private idiotope carried by 11-50. The G3 antibody had a strong inhibitory activity towards the binding of the 11-50 antibody with the Lc4 antigen, indicating that this anti-Id antibody reacts with the antigen binding site of the 11-50 antibody; i.e., the G3 antibody is a paratope-related anti-Id antibody (Fig. 4).

Fig. 4

When the reactivity of the G3 antibody towards the 11-50 antibody was analyzed by Western blotting technique, the results indicated that it recognized the idiotope which was formed by both heavy and light chains of the 11-50 antibody, and that it was not reactive with separated heavy or light chains of the antibody (data not shown).

When human polyclonal anti-Lc4 antibodies were purified from pooled human sera by Lc4-affinity chromatography, essentially all of the anti-Lc4 antibodies were recovered in the adsorbed fraction, when tested by ELISA using the immobilized Lc4 antigen. The purified human polyclonal anti-Lc4 antibodies were then tested for the reactivity with the G3 antibody as well as the reactivity with the Lc4 antigen in a sandwich assay system using immobilized neutral glycolipids from LS174T cells and the biotinylated G3 antibody. As shown in Fig. 5, significant reactive materials were detected only in the adsorbed fraction and not in the pass-through fraction. These reactive materials turned out to be mostly IgM accompanied by a small amount of IgG, when ascertained with goat anti-human  $\mu$ - and  $\gamma$ -chain specific antibodies. These results indicate that the G3 idiotope is not a private idiotope, but is a shared crossreactive idiotope, and that the pooled normal human sera contain a certain amount of the anti-Lc4 antibody which shares the G3-idiotope with 11-50.

Fig. 5



*Nucleotide and amino acid sequences of immunoglobulin variable regions*

Fig. 6 shows the nucleotide and deduced amino acid sequences of the immunoglobulin heavy chain variable regions of 11-50 and HMST-1. The homology between the V<sub>H</sub> segments of 11-50 and HMST-1 was high (93.5% at the nucleic acid level). The V<sub>H</sub> segments of both antibodies utilized the V<sub>H</sub>III family genes. Comparison of these V<sub>H</sub> segments with the known germline genes in data banks indicated that the V<sub>H</sub> segment of 11-50 had the highest homology to the germline gene V<sub>H</sub>1.9III (95.6% at the nucleic acid level), and that of HMST-1 to the germline gene hv3005f3 (98.3% at the nucleic acid level) (Berman *et al.*, 1988; Olee *et al.*, 1991). At the amino acid level, the V<sub>H</sub> segment of 11-50 was 95.6% homologous to V<sub>H</sub>1.9III, and that of HMST-1 was 97.3% homologous to hv3005f3. The D segments of these two antibodies could not be specified to any of the D genes known so far (Buluwela *et al.*, 1988; Ichihara *et al.*, 1988; Matsuda *et al.*, 1990). The J<sub>H</sub> segments of both antibodies were encoded by J<sub>H</sub>4.

Fig. 6

The V<sub>λ</sub> segments of these two antibodies also had a high homology (96.3% at the nucleic acid level), and were encoded by the V<sub>λ</sub> gene members which belong to the V<sub>λ</sub>II family (Fig. 7). The absence of sufficient germline sequence data in data banks made it impossible to determine the exact germline counterparts of these V<sub>λ</sub> segments. Among the rearranged V<sub>λ</sub> genes ever reported, V<sub>λ</sub>II.DS (Paul *et al.*, 1991) had the highest homology to the V<sub>λ</sub> segments of 11-50 and HMST-1. The V<sub>λ</sub> segments of both 11-50 and HMST-1 are 96.6 % homologous to V<sub>λ</sub>II.DS at the nucleic acid level. At the amino acid level, they were 92.7% and 94.5% homologous to V<sub>λ</sub>II.DS, respectively. The J<sub>λ</sub> segments of 11-50 and HMST-1 were encoded by J<sub>λ</sub>2 and J<sub>λ</sub>3, and amino acid sequence of the J<sub>λ</sub> segments of the two antibodies were identical.

Fig. 7

A few somatic mutations seemed to occur sporadically both in the heavy and light chain variable regions of these antibodies. These somatic mutations were observed both in the framework regions (FR) and the complementarity-determining-regions (CDR).



## DISCUSSION

The Lc4 antigen is known to be frequently expressed in various human cancer cells. In this paper we established the human monoclonal antibody 11-50 directed to Lc4, and the murine monoclonal anti-idiotypic antibody, G3, directed to an idiotypic carried by the 11-50 antibody. The G3 idiotypic was shown to be shared by at least a part of human polyclonal anti-Lc4 antibodies in pooled human sera.

At least some of the antibodies directed to carbohydrate antigens are known to be oligoclonal and to have a high idiotypic connectivity. For example, in the murine system, anti-idiotypic antibodies raised against antibodies toward onco-developmental carbohydrate antigen SSEA-1 (Le<sup>X</sup>) and related antigens react with other antibodies recognizing original antigens (Hirashima *et al.*, 1990; Umeda *et al.*, 1986). Approximately 50 % of the murine anti-SSEA-1 (anti-Le<sup>X</sup>) antibodies share the 6B1 idiotypic (Umeda *et al.*, 1986) and about 66 % of the anti-fucosyl SSEA-1 (anti-Le<sup>Y</sup>) antibodies carry the common B4 idiotypic (Hirashima *et al.*, 1990). In humans, it had already been shown as early as in 1968 that most of the human antibodies directed to both I- and i-antigens carry common cross-reactive idiotypes (Williams *et al.*, 1968), and more recently, human antibodies against capsular polysaccharide of *Haemophilus influenzae* were shown to share cross-reactive idiotypes (Lucas, 1988).

Regarding the molecular basis of idiotypic connectivity of antibodies, it is considered that antibodies are encoded by the same or closely related immunoglobulin variable region genes (Pascual *et al.*, 1991; Chen *et al.*, 1985). As to the murine monoclonal antibodies against onco-developmental carbohydrate antigens, the variable regions of most anti-SSEA-1 (anti-Le<sup>X</sup>) and anti-SSEA-3 monoclonal antibodies are known to be encoded by V<sub>H</sub>441 (Zenita *et al.*, 1988; Kimura *et al.*, 1989; Snyder *et al.*, 1990). The variable regions of most anti-fucosyl SSEA-1 (anti-Le<sup>Y</sup>) antibodies are coded by V<sub>H</sub>50.1 and V<sub>K</sub>K1A5 (Zenita *et al.*, manuscript in preparation). The idiotypic connectivity of human anti-Lc4 antibodies

suggests that these antibodies are encoded also by a restricted set of variable region genes, and that the G3 antibody recognizes the idiotype which is closely associated with these particular genes.

We tried to examine if the usage of the variable regions of human monoclonal antibodies against onco-developmental antigens are restricted, as in the case of murine antibodies (Zenita *et al.*, 1988; Kimura *et al.*, 1989; Snyder *et al.*, 1990). The  $V_H$ ,  $J_H$ ,  $V_L$  and  $J_L$  regions of the two human anti-Lc4 antibodies were encoded by a set of highly homologous genes. As to the CDR3 region of heavy chains, the D segments of the two antibodies have quite different amino acid sequence, though their lengths are the same. This situation is very similar to the case of murine anti-SSEA-1 and anti-fucosyl-SSEA-1 antibodies, in which case the  $V_H$ ,  $J_H$ ,  $V_L$  and  $J_L$  were highly homologous, with only the D regions significantly different (Kimura *et al.*, 1989; Zenita *et al.* manuscript in preparation). Surprisingly, in spite of the homology of the variable regions of 11-50 and HMST-1, the anti-idiotypic antibody G3 reacted only with 11-50 and the anti-Lc4 antibodies detected in pooled normal human sera, not with HMST-1. This lack of crossreactive G3 idiotype shared by 11-50 and polyclonal anti-Lc4 antibodies of HMST-1 may be under the influence of the differences of the CDR 3 region of the heavy chain from that of 11-50, or due to the somatic mutations in other regions. Since G3 recognizes the idiotope formed by both heavy and light chains, as previously described, and it reacted also with polyclonal anti-Lc4 antibodies, it is not considered that G3 idiotype is located solely in the  $V_H$  CDR3 region of 11-50. Considering the fact that 11-50 is derived from lymphocytes from a tonsil of a patient with non-malignant disease, and HMST-1 from regional lymph node cells of a cancer patient, it is interesting to speculate that HMST-1 lost the cross-reactive G3 idiotype of anti-Lc4 natural antibodies through the somatic mutations acquired during the course of the disease. The exact amino acid differences that can explain the difference of the reactivities against the anti-idiotypic antibody is currently unknown, but site-directed mutagenesis study can help to solve the problem.



The V<sub>H</sub> regions of 11-50 and HMST-1 are highly homologous to V<sub>H</sub>1.9III and hv3005f3 germline genes, respectively. V<sub>H</sub>1.9III and hv3005f3 are members of the closely related V<sub>H</sub>III germline genes and 98.6% homologous at the nucleic acid level (Olee *et al.*, 1991). An important point to note is that some members of V<sub>H</sub>III segments are preferentially utilized in the human fetal repertoire, and that rearranged immunoglobulin heavy chain genes FL2-2 and M72 which have been isolated from fetal liver are encoded by V<sub>H</sub>1.9III and hv3005f3 genes with only one or without any mutation, respectively (Schroeder *et al.*, 1987; Nickerson *et al.*, 1989; Schroeder and Wang, 1990). It is known that some members of the immunoglobulin variable genes are selectively rearranged in fetal B cells (Perlmutter *et al.*, 1985; Yancopoulos *et al.*, 1988). The V<sub>H</sub>1.9III and hv3005f3 genes are most probably examples of such genes which are preferentially rearranged in fetal B cells. Besides, they are also used by natural autoantibodies (Siminovitch and Chen, 1990).

It is reported from the studies using murine anti-Lc4 antibodies that the Lc4 antigen is strongly expressed in the embryonic and fetal periods (Rettig *et al.*, 1985; Williams *et al.*, 1982; Karlsson and Larson, 1979). In the sterile fetal environment, immature B cells would not be frequently stimulated by exogenous antigens, but the embryonic carbohydrate antigens including Lc4 as well as other autoantigens may stimulate a subset of primitive B cells expressing a preferentially-rearranged immunoglobulin variable region genes. This subset of B cells could be the producers of such natural autoantibodies. It would be interesting to speculate that a similar B cell subset would be stimulated and produce anti-Lc4 antibodies when the Lc4 is expressed on cancer cells in adults, as in the case of IgM<sup>WOO</sup> (Kabat *et al.*, 1982). In the case of IgM<sup>WOO</sup>, a patient with bronchogenic carcinoma, who showed an unusually favorable clinical course, was reported to have a monoclonal anti-Lc4 antibody in his serum, and the antibody was suggested to be involved in the immune reaction towards the cancer cells.

With regard to the light chains, all three human anti-Lc4 antibodies known to date have  $\lambda$ -light chains. We clarified here that V $\lambda$  of 11-50 and HMST-1 are



encoded by the same or closely related genes. Again they show very high homology to a rearranged V $\lambda$  gene V $\lambda$ II.DS which was utilized in autoantibody associated with lupus erythematosus. Whether the germline gene for V $\lambda$ II.DS is preferentially used in the fetal repertoire is currently unknown.

In summary, the immunoglobulin variable regions of the two human antibodies recognizing Lc4 are highly homologous to each other and to closely related germline genes which are also used in the fetal repertoire and in coding autoantibodies.

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## FIGURE LEGENDS

Figure 1. TLC-immunostaining of the neutral glycolipids prepared from human colon cancer cell line LS174T. Lane 1, orcinol/H<sub>2</sub>SO<sub>4</sub> staining which visualizes all glycolipids. Lane 2, TLC-immunostaining with the monoclonal antibody 11-50. Lane 3, TLC-immunostaining with the monoclonal antibody HMST-1. The solvent system for TLC was chloroform/ methanol/ 0.5% CaCl<sub>2</sub> in H<sub>2</sub>O (60:30:8, v/v/v).

Figure 2. Specificity of the monoclonal antibody 11-50 against various standard glycolipids as ascertained by ELISA. ●, Lc<sub>4</sub>; ○, asialo-GM<sub>1</sub> (Gg<sub>4</sub>); ▲, SSEA-3 (IV<sup>3</sup>GalβGb<sub>4</sub>); △, nLc<sub>4</sub> (paragloboside), asialo-GM<sub>2</sub>(Gg<sub>3</sub>), amino-CTH (Lc<sub>3</sub>), GM<sub>3</sub>, IV<sup>2</sup>FuconLc<sub>4</sub> (H1-glycolipid) and IV<sup>3</sup>GalβnLc<sub>4</sub> (Kraus-glycolipid). Each well of the 96-well plate was coated with 10 ng of purified glycolipids, 50 ng of phosphatidylcholine, and 25 ng of cholesterol. Reactivity was determined with serial dilution of the culture supernatant of the hybridoma 11-50, as indicated on the abscissa. Ordinate, absorbance at 500 nm.

Figure 3. Reactivity of monoclonal antibodies 11-50 (●) and HMST-1 (▲) toward purified Lc<sub>4</sub> antigen as ascertained by ELISA. Each well of the 96-well plate was coated with 10 ng of Lc<sub>4</sub> glycolipid, 50 ng of phosphatidylcholine, and 25 ng of cholesterol. Reactivity was determined with serial dilution of the respective purified antibodies as indicated on the abscissa (dilution starting from 20 µg/ml). Ordinate, absorbance at 500 nm.

Figure 4. Inhibitory activity of G3 toward the binding of 11-50 with Lc4 antigen. Each well of the 96-well plate was coated with 20ng of Lc4 glycolipid, and 11-50 was added to the well in the presence of G3 (●) or isotype matched control antibody 15B (▲). Reactivity was determined with serial dilution of respective purified antibodies as indicated on the abscissa (dilution starting at 10 µg/ml). Ordinate, absorbance at 500nm.

Figure 5. Idiotype positive anti-Lc4 components in pooled normal human sera. Pooled normal human sera were applied to Lc4-affinity column chromatography. O—O, titer of 11-50-like antibody as ascertained by ELISA using immobilized neutral glycolipids of LS174T cells which contains Lc4 antigen and the biotinylated anti-idiotypic monoclonal antibody, G3; — absorbance at 280 nm.

Figure 6. The nucleotide and deduced amino acid sequences of the 11-50 and HMST-1 heavy chain variable regions. The V<sub>H</sub> regions are compared to the germline V<sub>H</sub>1.9III (Berman *et al.*, 1988) and hv3005f3 genes (Olee *et al.*, 1991), which showed the highest homology among the genes known so far. The J<sub>H</sub> regions are compared with J<sub>H</sub>4. Nucleotides identical to those of V<sub>H</sub>1.9III are indicated by dashes. The deduced amino acid sequences are shown in one letter code, and the start sites for each CDR and FR region are indicated. Amino acids are numbered, and CDR as well as FR regions are defined according to Kabat *et al.* (1987).

Figure 7. The nucleotide and deduced amino acid sequences of the 11-50 and HMST-1 light chain variable regions. The V<sub>λ</sub> regions are compared to the V<sub>λ</sub>II.DS rearranged gene, which showed the highest homology to both V<sub>λ</sub> segments. The J<sub>λ</sub> regions are compared with J<sub>λ</sub>2 and J<sub>λ</sub>3. Amino acid sequences are numbered, and CDR as well as FR regions are defined according to Chuchana *et al.* (1990). N, unspecified nucleotide; X, unspecified amino acid.



Fig. 1

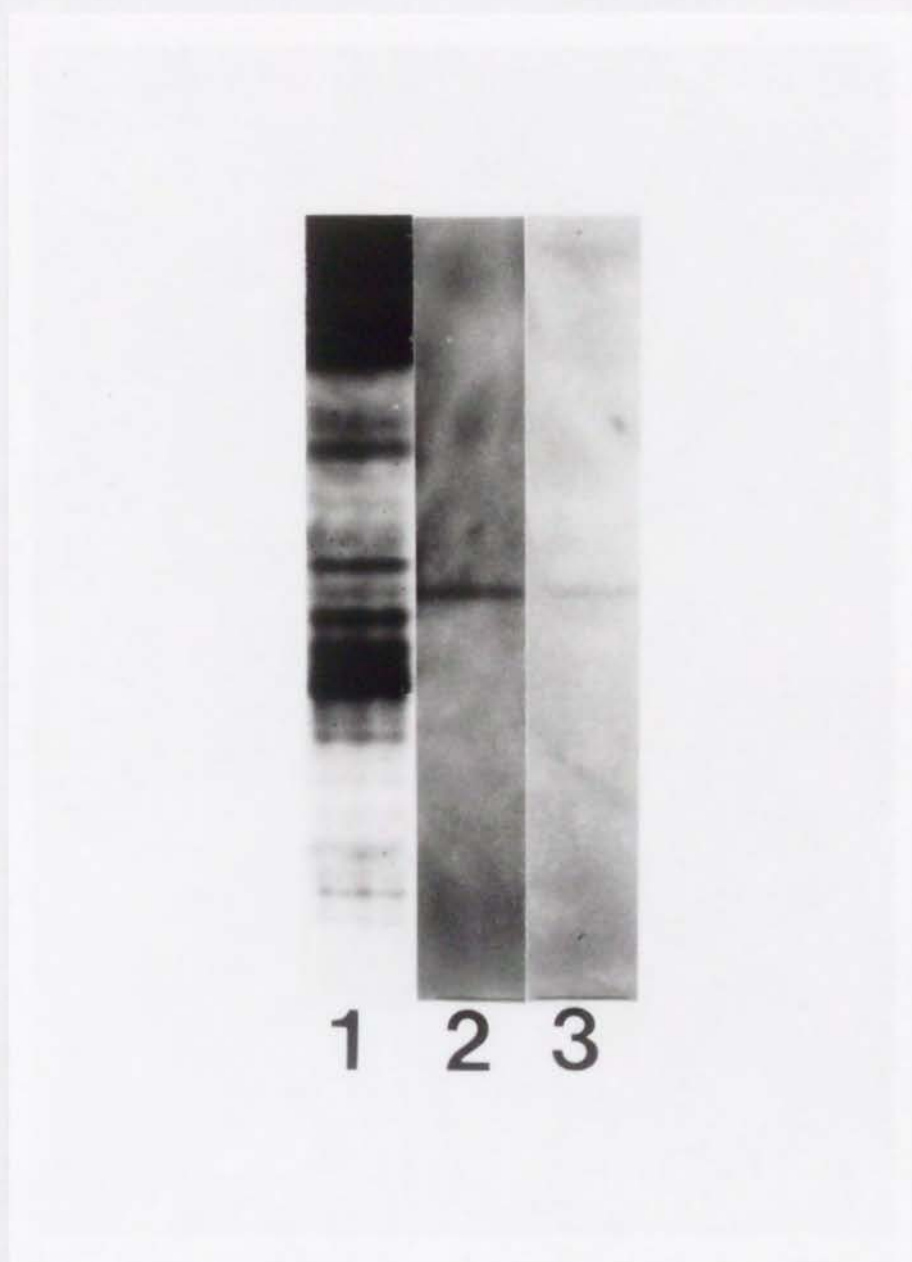


Fig. 2

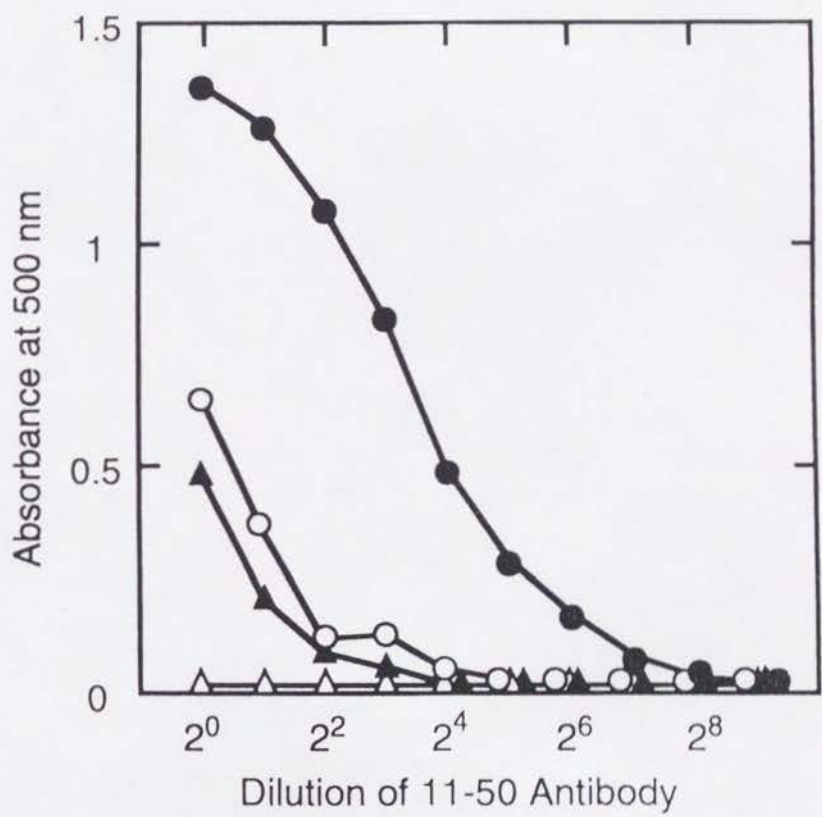


Fig. 3

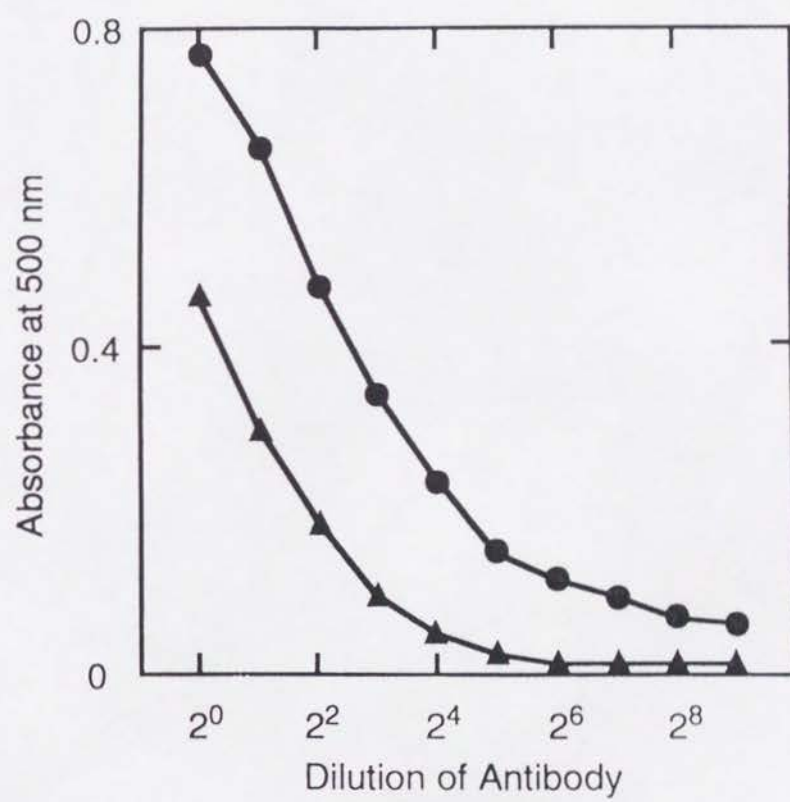




Fig. 4

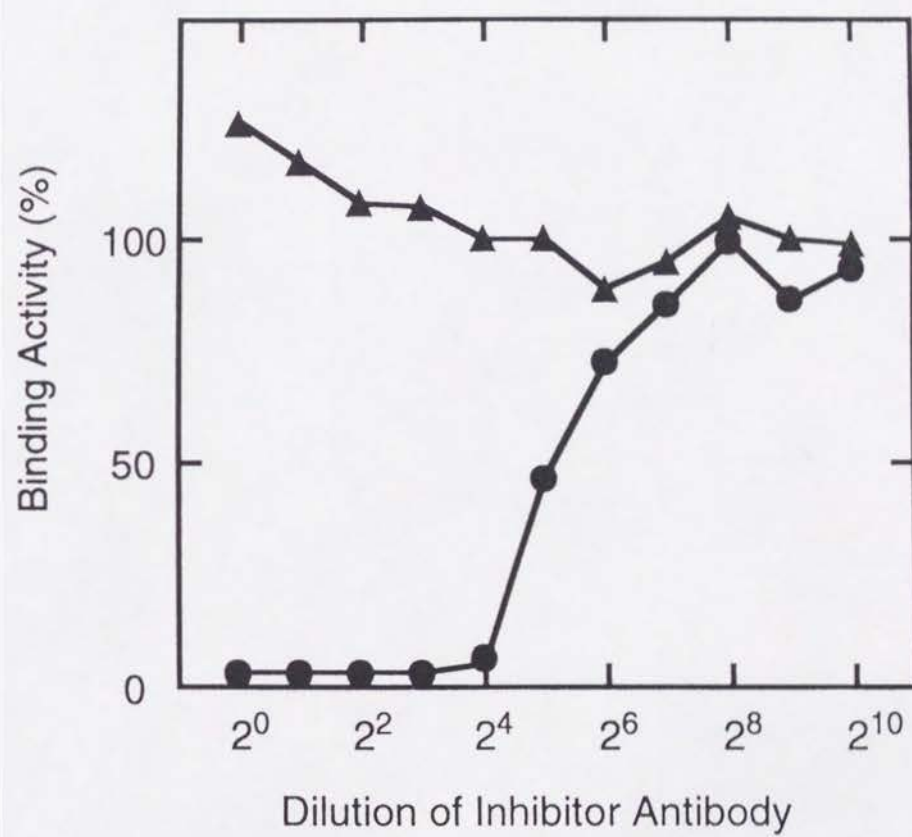


Fig. 5

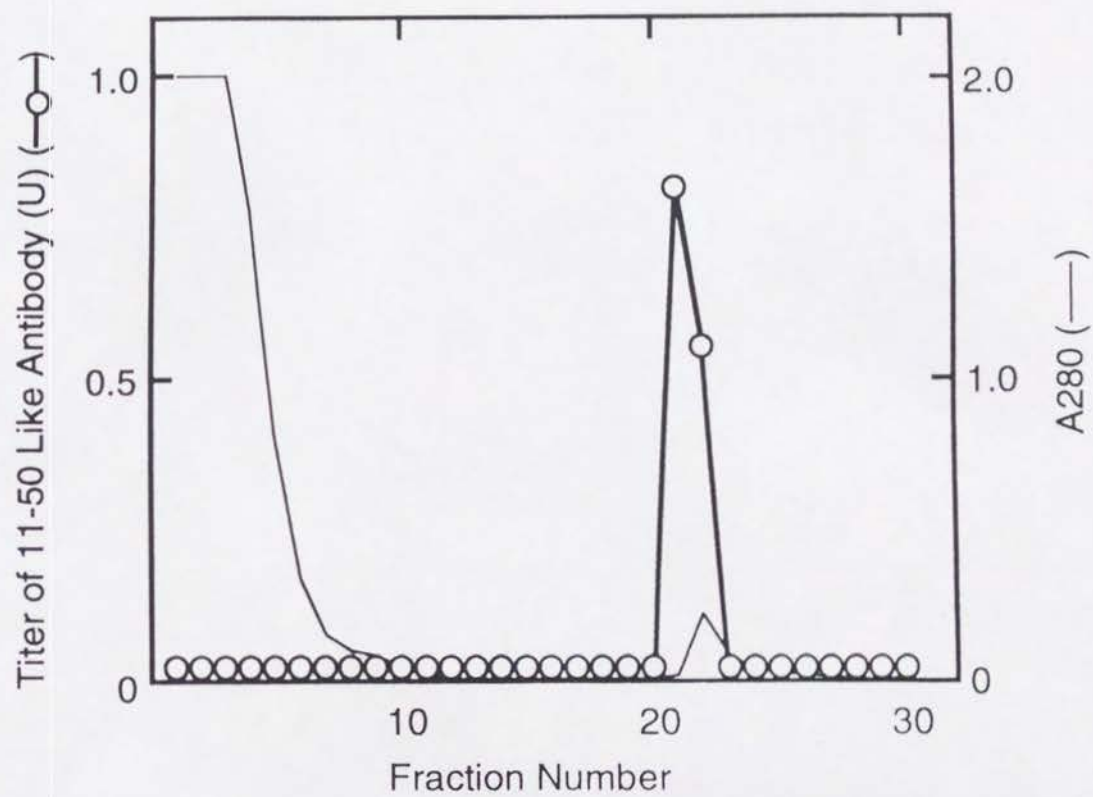


Fig. 6

[illegible]



Fig. 7

VALI.DS 1 FRI 10 20 30  
 CAGTCTGCCCTGACTCAGCCTGCCCTCCGTGCTGGGCTCCTGGACAGTCGATCACCATTCTCCTGCACCTGGAACCAAGCAGTGACGTTGGT  
 11-50 Q S A L T Q P A S V S G S P G Q S I T I S C T G T S S D V G  
 N-----NN-----  
 X X  
 HMST-1 N-----NN-----  
 X X  
 CDR1 FR2 40 CDR2 50 FR3 60  
 GGTATACTATGTCTCCTGGTACCAACAGCACCAGCTAAAGCCCCAACTCATGATTATGATGTCAAGTAAAGGCGCTCAGGGGTT  
 11-50 G Y N Y V S W Y Q Q H P A K A P K L M I Y D V S K R P S G V  
 A-----T-----GC-----T-----  
 S G V  
 HMST-1 A-----T-----TC-----T-----T-----C-----  
 V F N  
 VALI.DS 68 71 80 90  
 TCTATCGCTTCTCTGGCTCCAGTCTGCGCAACACGGCCTCCCTGCACCATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTA  
 11-50 S N R F S G S K S G N T A S L T I S G L Q A E D E A D Y Y C  
 G-----G-----G-----A-----  
 C V T  
 HMST-1  
 CDR3 101 103 FR4 110  
 AGTCATATACAGCAGCAGCAGCTTTC (J12,3) GTATTGCGGAGGAGGACCAAGCTGACCGTCCTAG  
 11-50 S S Y T S S S T F V F G G G T K L T V L  
 GG-----G-----T-----T-----A-----  
 M  
 HMST-1 G-----AGG-----  
 R R  
 C12 GTACGCCCAAGGCTGCCCTCGGTCATCTGTTCGCCGCCCTCCTGTGAGGAGCTTCAAGCCCAACAGGCCACACTG  
 11-50 G Q P K A A P S V T L F P P S S E E L HL FOR PRIMER  
 S  
 C13 A-----A-----  
 HMST-1 A-----